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PHENANTHRENE REMOVAL BY *PENICILLIUM FREQUENTANS* GROWN ON A SOLID-STATE CULTURE: EFFECT OF OXYGEN CONCENTRATION

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ABSTRACT

Phenanthrene removal by *Penicillium frequentans* was compared under aerobic and microaerophilic conditions in a solid culture amended with low quantities of an agricultural residue. An inoculum of *P. frequentans* grown on sugarcane bagasse pith was mixed with soil spiked with 200 mg l^{-1} of phenanthrene, to obtain a final bagasse/soil ratio of 1:16. The C/N ratio was adjusted to 60 and the moisture content to 40%. The oxygen concentrations were adjusted to 20%, 10%, 5%, 2% and close to 0%, in the soil-gas phase for each treatment. There were statistically significant ($p<0.05$) differences in the metabolic activity at different oxygen concentrations, measured as CO_2 production. Phenanthrene removal rates increased with oxygen concentration, reaching 52% removal after 17 days of incubation for the treatment with 20% O_2. Nevertheless, oxygen-limited (microaerophilic) conditions did not preclude phenanthrene degradation.

Keywords: Oxygen concentration, solid state-culture, *Penicillium frequentans*, PAHs, sugarcane bagasse pith

INTRODUCTION

Most polycyclic aromatic hydrocarbons (PAH), especially the highly condensed compounds with 4 or more rings, and their metabolites, are mutagenic and carcinogenic to microorganisms, plants and animals, and are classified as compounds with significant human health risk [1]. These compounds are produced at high-temperatures in industrial processes such as petroleum refining, coke production, wood burning and synthetic oil and gas production. PAH are associated with a wide range of hazardous waste sites [2].

PAH are relatively recalcitrant but losses are observed over time which may occur through biotic processes or a variety of abiotic processes including leaching, photodegradation and volatilization [3]. Another PAH removal mechanism is biodegradation. The efficacy of this process as a removal mechanism depends on the type and concentration of the PAH present, soil characteristics, moisture content, temperature, microbial diversity, type and balance of nutrients, electron receivers and soil gas oxygen concentrations [4]. However, the soil gas oxygen concentration is generally thought to be one of the limiting factors in PAH biodegradation, not only in its role as electron acceptor during microbial respiration but also because it serves as a co-substrate for oxygenase enzymes that initiate PAH catabolism [3, 5].

Due to the importance of oxygen in the dynamics of the aerobic processes in soil, many studies have been carried out to investigate the effect of oxygen concentration on the sequestration and bioavailability of diverse aromatic hydrocarbons in soil [6]. Oxygen concentration varies according to soil depth and its availability depends on the oxygen diffusion and consumption rates, which in turn depend on the type of soil and the presence of bioavailable substrates. These factors can therefore affect the efficacy of aerobic remediation processes of PAH-contaminated soils.

An aspect that is related to the process of oxygen diffusion in solid medium, is the addition of agroindustrial residue materials, which enhance considerably the porosity and facilitate air diffusion, increasing the gas exchange necessary for the aerobic metabolic process. Aerobic solid-state cultures offer several advantages for the treatment of hydrocarbon-contaminated soils, however, limited attention has been given to the effect of oxygen concentrations on process performance [7], and there is a lack of information regarding the bioremediation potential of solid-state cultures under microaerophilic conditions. This is important because bioremediation of organic soils contaminated by toxic

organics often involves the addition of bulking agents (e.g. agroindustrial residues) that enhance soil porosity and oxygen diffusion and serve as microbial carriers [8-10]. Nevertheless, these bulking agents also contribute to the oxygen demand of the system, which is conducive to the development of microaerophilic niches. Thus, it is important to evaluate the metabolic activity and PAH degradation capabilities of solid-state cultures at various oxygen concentrations. Studies using low amounts of agricultural residues to remove hydrocarbon compounds are scarce [11-13].

The aim of this study was to characterize the effect of low oxygen concentrations on phenanthrene removal in a solid-state culture using the fungus *Penicillium frequentans* grown on sugarcane bagasse since, when low amounts of this residue are used oxygen diffusion decreases. Different soil gas conditions were prepared to gain a better understanding of heterotrophic activity and phenanthrene removal by *P. frequentans* as a function of the oxygen concentration.

MATERIALS AND METHODS

Soil Characterization

Soil was obtained from an uncontaminated tropical site in the state of Tabasco, Mexico (18° 00′ 38″ E and 93° 05′ 02″ W). The soil was air-dried, homogenized (gently sieved) and characterized before treatment.

Evaluation of the soil characteristics and chemical properties showed that the texture was sandy clay loam with a slightly acid pH (5.7), which is adequate for fungal growth. The organic matter content was 6.8 % [11].

Fungal Inoculum Preparation in Solid-state Culture

The filamentous fungus *P. frequentans* was isolated from sugarcane bagasse pith [13]. Fungal mycelium was obtained by growing the fungus in modified Wunder medium in 250 ml Erlenmeyer flasks, in liquid culture at 28 °C, 120 rpm, for 4 days. The modified Wunder medium [14] had the following composition (g l^{-1}): glucose H_2O, 10.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$ $7H_2O$, 0.5; KH_2PO_4, 0.875; K_2HPO_4, 0.125; $CaCl_2.2H_2O$, 0.1; NaCl, 0.1; $FeSO_4$, 0.001; pH 5.5. Distilled water was used in the preparation of medium.

To obtain the fungus inoculum by solid culture, 0.02 g of mycelium in dry weight obtained from 4 days liquid culture were added to 0.48 g of sterilised sugarcane bagasse pith in a 125 ml sealed bottle and grown for 3 days. Sugarcane bagasse pith and soil were sterilized for three discontinuous days by autoclaving at 121°C/15 psi to ensure complete sterility and to avoid microbiological activity.

Different controls were employed in order to ascertain the abiotic removal by adsorption to soil and bagasse. The controls were as follows: sterilized soil and sterilized sugar-cane bagasse pith without fungus were used to ensure the absence of microbiological activity and sterilization efficiency was confirmed by the absence of carbon dioxide production; soil and sugar-cane bagasse pith without fungus and with phenanthrene, to determine the effect of abiotic removal; and sterilised soil plus sterilised sugar-cane bagasse pith with fungus and without phenanthrene to evaluate the basal microbial activity of the system (Figure 1).

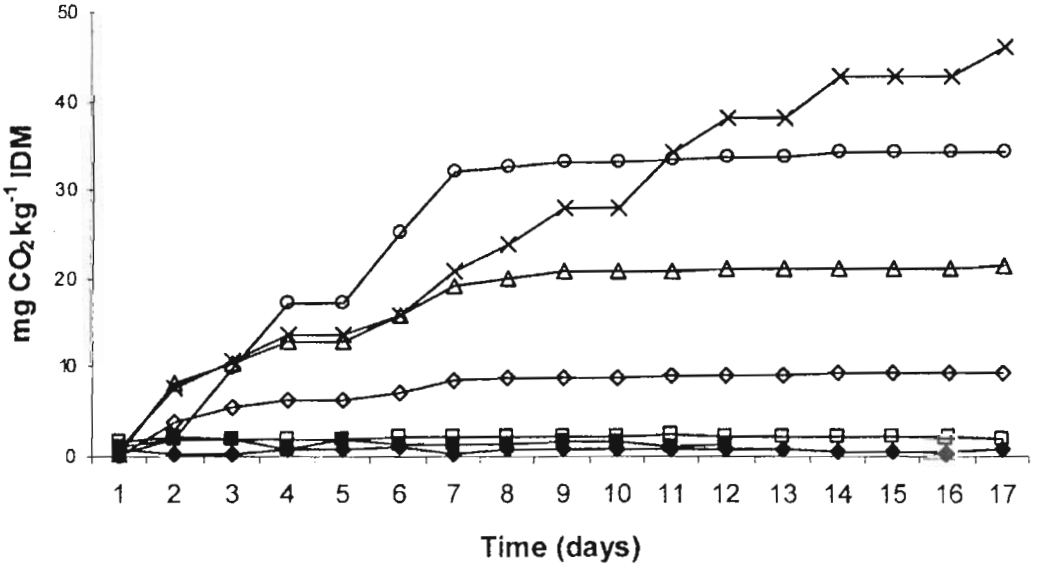

Figure 1. Heterotrophic activity measured as accumulated CO_2 production in a solid- state culture, under different O_2 concentrations. IDM=Initial dry matter: Ctrl Ab= Control soil and sugar-cane bagasse pith without fungus and with phenanthrene T= 28°C, M=40%, C/N=60, Rate soil residue = 96:4. O20% X10%, Δ5% ◊2% □ close to 0% ♦ ctrl ab ■ ctrl phe

Phenanthrene Removal at Different Oxygen Concentrations

6 g of sterilized soil spiked with phenanthrene were dissolved in acetone 24 h before inoculation, to allow solvent evaporation. The fungus inoculum was added to the soil spiked with the 200 mg l^{-1} of final phenanthrene solution, and kept in the dark for 17 days at 28° C. Subsequently, all the systems were mixed by vortex for 2 minutes in order to attain a homogeneous moisture content and a good distribution of bagasse and nutrients in the soil. Three controls were set up: sterilized soil amended with 200 mg kg^{-1} of phenanthrene to investigate phenanthrene removal by adsorption to the soil matrix; sterilised soil and bagasse to ensure sterilization of the process; and sterilised soil and bagasse with fungus and without phenanthrene, to establish the microbial activity of the system. Experiments were run in duplicate, except for the controls which were run in triplicate, while mixing in oxygen or nitrogen (99.9% pure), depending on the desired oxygen concentration in the soil gas. The oxygen concentration was adjusted by a daily supply of both gases, oxygen and nitrogen, for 20-90 seconds (depending on the oxygen adjustment) to obtain a final concentration of 20%, 10%, 5%, 2% and close to 0% (Henry´s constant for oxygen = 760 atm-L $mole^{-1}$). The dependent variables were: carbon dioxide evolution, oxygen concentration and phenanthrene removal on a dry weight basis. Average oxygen concentration was calculated, for each treatment, twice a day, before and after oxygen adjustment. Carbon dioxide evolution and oxygen concentration were monitored daily by gas chromatography using a syringe to extract the gas from the headspace of the treatment units as is described below.

Kinetics of Phenathrene Removal

Phenanthrene removal rates at different oxygen concentrations were measured for 17 days. Residual phenanthrene concentrations were evaluated on days 4, 7, 9, 11, 14 and 17 to determine the effect of oxygen concentration on the ability of the fungus to remove phenanthrene.

Carbon Dioxide Evolution and Oxygen Concentration During Treatment

Heterotrophic activity was measured by the indirect method of carbon dioxide evolution and quantified as mg CO_2 per kg of initial dry matter (IDM) [15, 16]. Oxygen consumption was measured to evaluate the oxygen concentration before and after treatment. Carbon dioxide concentration in the headspace was measured daily by gas chromatography immediately before oxygenation. These data were used to calculate the respiratory quotient (Equation i), which was obtained using the carbon dioxide evolved per mole of oxygen consumed, and provides a direct measurement of microorganism respiratory activity [17]. Respirometric values were determined by other authors and can vary from 0.67 to 5 depending on culture conditions [17]. Values greater than five indicate anaerobic conditions:

$$\text{Respiratory Quotient (RQ)} = \frac{CER}{OUR} \qquad \text{(i)}$$

where:
CER = *production of* CO_2 $(mol\ t^{-1}\ l^{-3})$
OUR = *consumption of* O_2 $(mol\ t^{-1}\ l^{-3})$

Headspace samples (1 ml) were taken with a 5 ml gas-tight syringe. The evolution of carbon dioxide and residual oxygen was determined from the headspace of vials, using a Gow-Mac 580 gas chromatograph equipped with a thermal conductivity detector and an Alltech CTRI stainless steel column. The operation conditions were: 25°C oven temperature, 40°C injector temperature and 100°C detector temperature. Helium was used as a carrier gas. Data were processed using Gow-Mac software and integrated to obtain the instantaneous carbon dioxide production and residual oxygen in percentage. Detection limits were 0.001% for both carbon dioxide and oxygen concentrations. For the experiment period the system was purged with oxygen and nitrogen gas for about 20 to 90 seconds, depending on each adjustment, to reach the desired soil-gas oxygen concentration, which was checked by gas chromatography analysis of the headspace gas. Soil controls were processed exactly as described above but without the addition of phenanthrene and without *P. frequentans*.

Phenanthrene Extraction and Analysis

Soil samples were removed from each bottle and extracted in a Soxhlet system using ketone as the solvent, for 8 h (EPA-3540).

Soil extracts were analyzed quantitatively by HPLC VARIAN 9012 equipment, fitted with a capillary column (Res Elut 5u C18 90Å, 4.6 mm id, 150 mm length) equipped with a UV/VIS 9050 detector. Samples were analyzed at 240 nm, methanol: water was used as the mobile phase in a 90:10 ratio and a flow velocity of 1.0.

Statistical Analysis

Regression analysis and Least Significant Difference (LSD) tests were performed through the use of the software SAS 6.08 (Statistical Analysis System). Data for oxygen consumption, carbon dioxide production, residual oxygen concentration, treatment time and phenanthrene removal percentages were compared by the LSD test at a 95% confidence level.

RESULTS AND DISCUSSION

Heterotrophic Activity of *P. frequentans* in a Solid-State Culture at Different Oxygen Concentrations

Figure 1 shows the heterotrophic activity of *P. frequentans* quantified as instantaneous carbon dioxide production. Oxygen concentrations were adjusted to 2%, 5%, 10%, and 20% in the soil gas phase, variations between treatments being lower than ±1% ($p < 0.005$). Although microaerophilic conditions were established (oxygen concentration < 2%) it was not possible to achieve 0% oxygen concentration (oxygen concentration measured in the headspace was in the order of 0.1-0.01%), due to the existence of a minimum amount of soil gas oxygen in the solid-state culture. This could be due to traces of oxygen present in porous microspaces or soil clay-colloids inside the solid culture [18].

Respirometric values were high from day 1 to day 3, before phenanthrene and soil addition (Figure 1). This was due to both biostimulation by the nutrients added and the assimilation of bagasse carbohydrates, which may enhance the heterotrophic activity [19, 20]. The increase in carbon dioxide production after phenanthrene and soil addition indicates that the fungus was able to utilize the organic matter and nutrients of the soil.

Figure 3 shows the respiratory quotient values obtained from the evolution of carbon dioxide and the amount of consumed oxygen at each oxygen concentration. For all treatments, the respiratory quotient was consistently below two units and it decreased with an increase in oxygen concentration (*i.e.* less anaerobic), which is consistent with lower concentrations observed for quotients close to two units. This indicates that some of the organic compounds

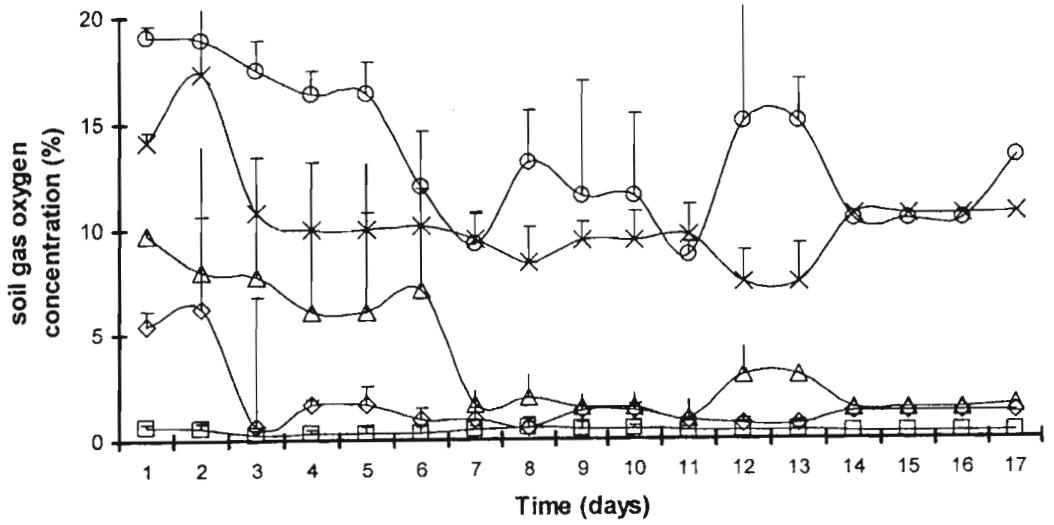

Figure 2. Residual oxygen concentration during the treatment in a solid culture. Third day indicates the time when polluted soil was added. Temp= 28°C, M =40%, C/N=60, Rate soil :residue=96:4. O20% X10%, Δ 5% ◊ 2% □close to 0%.

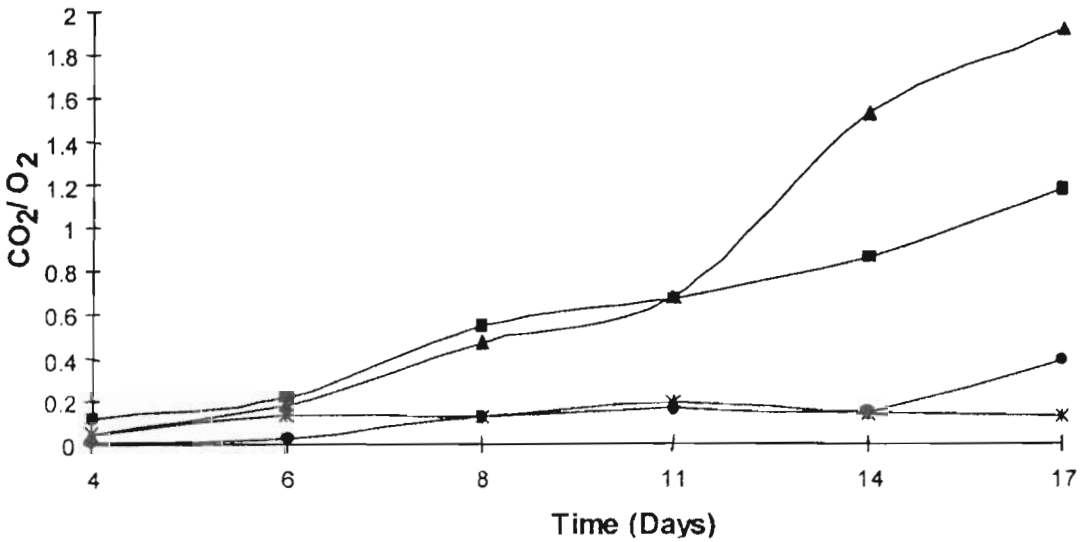

Figure 3. Respiratory quotient CO_2/O_2, calculated from the heterotrophic activity of *P. frequentans* at different oxygen levels.

from the soil and bagasse were oxidized to carbon dioxide and water, under aerobic conditions [17], which was confirmed by the controls (fungus grown on sterilized soil and fungus grown in sterilized bagasse) that were used to determine CO_2 production from soil organic matter and bagasse (Figure 1), respectively. A low CO_2 production was observed, which was 4 to 10% above the other two controls (sterilized soil and sterilized bagasse without fungus), for which CO_2 production was not observed.

Determination of Phenanthrene Removal by *P. frequentans* in a Solid-state Culture at Different Oxygen Concentrations

Regression analysis revealed that treatment time and oxygen concentration had a statistically significant effect on phenanthrene removal ($p<0.0003$ and $p<0.0001$ respectively). However, the interaction of these two variables did not show a significant effect ($p<0.05$) on phenanthrene removal (Table 1).

Values for CO_2 evolution and oxygen concentration at all levels of oxygen showed significant differences ($p<0.005$) between treatments. Phenanthrene removal increased with oxygen concentration and treatment time. The highest phenanthrene removal was observed at oxygen concentrations of 20% and 10% on day 17 (Figure 4). Nevertheless, phenanthrene removal occurred under microaerophilic conditions (oxygen concentration < 2%), and showed slower rates than when oxygen was not limiting.

Table 1. Regression analysis of CO_2 evolution (mg CO_2 kg^{-1} IDM).

Term	DF	*F* Value	Significance level
Time of treatment	5	7.30	0.0003
O_2 concentration	4	15.16	0.0001
Time of treatment * O_2 concentration	20	1.49	0.1608

Significantly high values; $r^2 = 0.8194$; Coefficient of variation = 37.54; $\alpha = 0.05$; Confidence = 0.95; D.F.= Degree of freedom; *F*=Fisher value; IDM= Initial dry matter

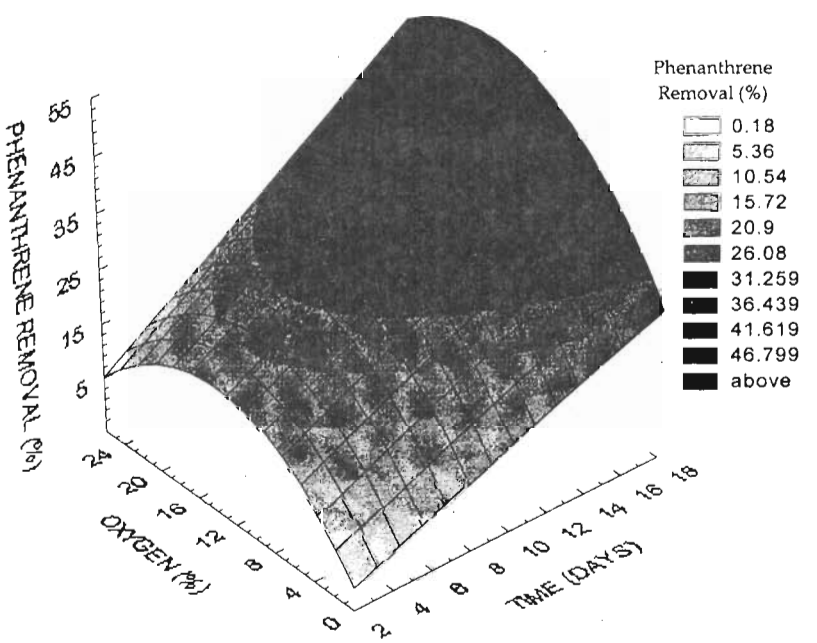

Figure 4. Surface response graph of phenanthrene by *P. frequentans* removal as a function of oxygen concentrations and treatment time.

For example, 13% of phenanthrene removal was obtained at close to 0% percent of oxygen on day 17. This is possible since anoxic conditions were not reached, as stated above, and a minimal soil gas oxygen concentration was present, as required for the above mentioned 13% phenanthrene removal at 0% of oxygen. This agrees with results obtained in liquid culture [21], where microaerophilic conditions did not hinder the organic compounds removal in the liquid culture. The critical oxygen level required to induce aerobic biodegradation varies with microbial population and substrate and can range from 0.013 to 1.5 mg O_2 l^{-1} [22] and a minimum oxygen concentration in the range of 0.01 to 0.038 mg l^{-1} has been reported for some bacterial and yeast cultures [23]. Other authors [24] reported a critical dissolved oxygen concentration of 0.35 mg l^{-1} for *Azotobacter vinlandii* and 0.12 for *Escherichia coli* needed for the expression of toluene dioxygenases for benzene, toluene and xylene degradation [22, 25].

Past studies have also emphasized the need for a minimum concentration of dissolved oxygen for the oxygenase-mediated biodegradation of monoaromatic hydrocarbons, particularly benzene, which is relatively recalcitrant under anaerobic conditions. Apparently, the synthesis and activity of oxygenase enzymes responsible for the initial attack on the aromatic ring is regulated by oxygen concentration [2, 26]. However, in this study, as in liquid culture [22-24], a minimal concentration of oxygen may be required to activate the fungal enzymatic activity for phenanthrene removal. Studies carried out under microaerophilic conditions have often reported that oxygen concentration can influence other processes that affect contaminant degradation kinetics. Thus, it appears that slower oxygen diffusion can limit metabolic processes that affect degradation rates [21, 27].

Results for the kinetics of phenanthrene removal showed significant differences ($p<0.005$) in phenanthrene removal at oxygen values close to 0 and 2%. On day 17, phenanthrene removal in samples with 20%, 10% and 5% of oxygen became significantly higher than those with 2% oxygen. Figure 5 shows significant phenanthrene removals of 13% and 11% for oxygen values close to 0% and for the abiotic control, respectively, which suggests the occurrence of important physical-chemical processes, like adsorption-desorption, specifically in the abiotic treatment. A similar result has been observed during soil bioremediation for the degradation of hydrophobic compounds, like phenanthrene and other non-polar compounds, whose concentration decreased due to their adsorption on the organic fraction of soil [28, 29]. The adsorption/desorption process is increased when soil has a high moisture content because solutes adsorbed to organic matter are significant, thus the sequestration of phenanthrene by soil organic matter is fast [30, 31].

CONCLUSIONS

Oxygen concentration has a significant effect on phenanthrene removal. A high removal rate was obtained for

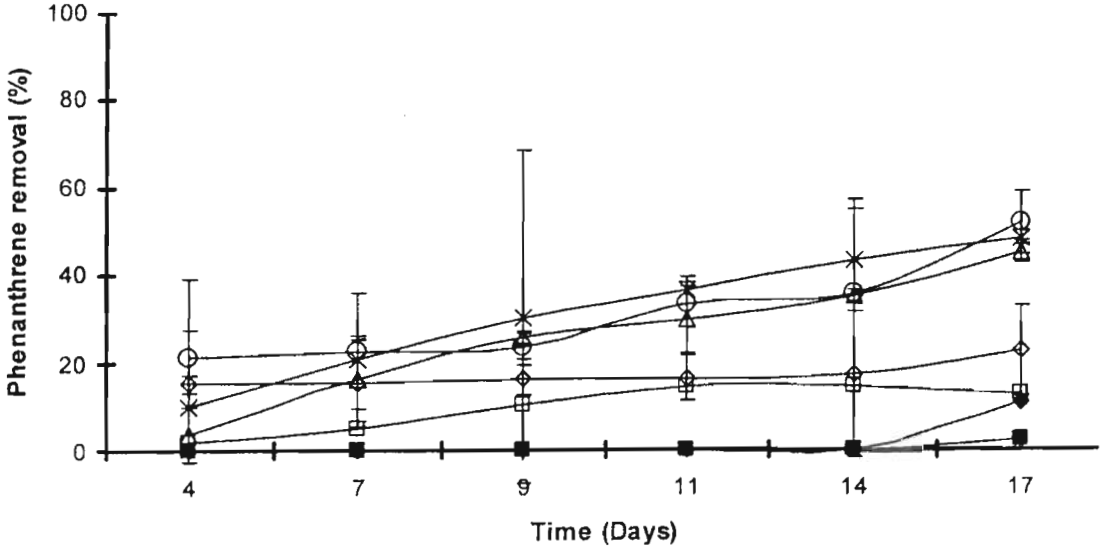

Figure 5. Kinetics for phenanthrene removal at different oxygen concentrations by *P. frequentans* at different oxygen concentrations. ctrl phe= sterilized oil plus sterilized sugar-cane bagasse pith with fungus and without phenanthrene O 20% X 10%, Δ 5% ◊ 2% □ close to 0% ♦ ctrl ab ■ ctrl phe

the higher oxygen concentrations and after 17 days of treatment, and an opposite tendency was found under low oxygen conditions and at the time treatment commenced.

Microaerophilic conditions did not preclude phenanthrene removal, reaching 13% at close to 0% of oxygen concentration at day 17. This may be explained by adsorption of soil components since the organic matter (6.3%) and clay contents were high.

In addition, these results partly explain the relatively high percentages of hydrocarbon removal in systems where low amounts of agricultural residues (2% w/w) have been added. These materials offer advantages over inert materials for hydrophobic compounds removal since they can provide a support and carbon source for microbial growth and they can increase the soil porosity and surface area, which could enhance both the gas diffusion and adsorption of organic compounds.

These results are of interest in soil bioremediation because of the lack of information on degradation of toxic organic compounds under limited oxygen supply or microaerophilic conditions in solid culture, since most of the information available is related to systems under saturated oxygen conditions.

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